Buchwald, S. L., & Knowles, J. R. (1980) *J. Am. Chem. Soc. 102*, 6601–6603.
Buchwald, S. L., Hansen, D. E., Hassett, A., & Knowles, J. R. (1982) *Methods Enzymol. 87*, 279–301.
Cohn, M. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 23–42.
Eckstein, F. (1983) *Angew. Chem., Int. Ed. Engl. 22*, 423–439.
Eckstein, F., Romaniuk, P. J., & Connolly, B. A. (1982) *Methods Enzymol. 87*, 197–212.
Eibl, H. (1980) *Chem. Phys. Lipids 26*, 405–429.
Floss, H. G., Tsai, M.-D., & Woodard, R. W. (1984) *Top. Stereochem. 15*, 253–321.
Frey, P. A. (1982) *Tetrahedron 38*, 1541–1567.
Frey, P. A., Richard, J. P., Ho, H.-T., Brody, R. S., Sammons, R. D., & Sheu, K.-F. (1982) *Methods Enzymol. 87*, 213–235.
Gerlt, J. A., Coderre, J. A., & Mehdi, S. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol. 55*, 291–380.
Hanahan, D. J., & Chaikoff, I. L. (1947a) *J. Biol. Chem. 168*, 233–240.
Hanahan, D. J., & Chaikoff, I. L. (1947b) *J. Biol. Chem. 169*, 699–705.
Hanahan, D. J., & Chaikoff, I. L. (1948) *J. Biol. Chem. 172*, 191–198.
Heller, M. (1978) *Adv. Lipid Res. 16*, 267–326.
Heller, M., Mozes, N., & Maes, E. (1975) *Methods Enzymol. 35*, 226–232.
Jiang, R.-T., Shyy, Y.-J., & Tsai, M.-D. (1984) *Biochemistry* (following paper in this issue).
Kanfer, J. N. (1980) *Can. J. Biochem. 58*, 1370–1380.
Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877–919.
Lowe, G., Cullis, P. M., Jarvest, R. L., Potter, B. V. L., & Sproat, B. S. (1981) *Philos. Trans. R. Soc. London, Ser. B. 293*, 75–92.
Tsai, M.-D. (1979) *Biochemistry 18*, 1468–1472.
Tsai, M.-D. (1982) *Methods Enzymol. 87*, 235–279.
Tsai, M.-D., & Bruzik, K. (1983) *Biol. Magn. Reson. 5*, 129–181.
Tsai, M.-D., Huang, S. L., Kozlowski, J. F., & Chang, C. C. (1980) *Biochemistry 19*, 3531–3536.
Tzur, R., & Shapiro, B. (1972) *Biochim. Biophys. Acta 280*, 290–296.
Webb, M. R. (1982) *Methods Enzymol. 87*, 301–316.
Yang, S. F. (1969) *Methods Enzymol. 14*, 208–211.
Yang, S. F., Freer, S., & Benson, A. A. (1967) *J. Biol. Chem. 242*, 477–484.

Phospholipids Chiral at Phosphorus. Absolute Configuration of Chiral Thiophospholipids and Stereospecificity of Phospholipase D†

Ru-Tai Jiang, Yeun-Jund Shyy, and Ming-Daw Tsai*

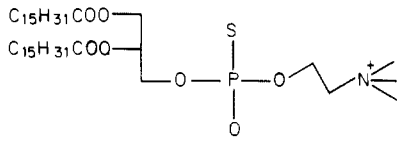
ABSTRACT: Separate diastereomers of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine (DPPsE) were prepared in 97% diastereomeric purity and characterized by ^{31}P, ^{13}C, and ^{1}H nuclear magnetic resonance (NMR). The isomers hydrolyzed by phospholipases A_2 and C specifically were designated as isomer B (^{31}P NMR δ 59.13 in $CDCl_3$ + Et_3N) and isomer A (59.29 ppm), respectively, analogous to the isomers B and A of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC) [Bruzik, K., Jiang, R.-T., & Tsai, M.-D. (1983) *Biochemistry 22*, 2478–2486]. Phospholipase D from cabbage was shown to be specific to isomer A of DPPsC in transphosphatidylation. The product DPPsE was shown to be isomer A. The absolute configuration of chiral DPPsE at phosphorus was elucidated by bromine-mediated desulfurization in $H_2{}^{18}O$ to give chiral 1,2-dipalmitoyl-*sn*-glycero-3-[^{18}O]phosphoethanolamine ([^{18}O]DPPE) followed by ^{31}P NMR analysis [Bruzik, K., & Tsai, M.-D. (1984) *J. Am. Chem. Soc. 106*, 747–754]. The absolute configuration of chiral DPPsC was elucidated by desulfurization in $H_2{}^{18}O$ mediated by bromine or cyanogen bromide to give chiral 1,2-dipalmitoyl-*sn*-glycero-3-[^{18}O]phosphocholine ([^{18}O]DPPC), which was then converted to [^{18}O]DPPE by phospholipase D with retention of configuration [Bruzik, K., & Tsai, M.-D. (1984) *Biochemistry* (preceding paper in this issue)]. The results indicate that isomer A of both DPPsE and DPPsC is S_P whereas isomer B is R_P.

Following the development of various nucleoside phosphorothioates (Eckstein, 1983), some thiophospholipids have been synthesized (Nifant'ev et al., 1978; Chupin et al., 1979) and used in biochemical and biophysical studies (Bruzik et al., 1982, 1983; Tsai et al., 1983; Vasilenko et al., 1982; Orr et al., 1982; Hui et al., 1983). However, most of the earlier work dealt with a diastereomeric mixture of thiophospholipids. Recently, we have reported (Bruzik et al., 1982, 1983) that 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine (DPPsC)[1] (**1**) exists in two diastereomers designated as isomer A (^{31}P NMR δ 56.12 in $CDCl_3$) and isomer B (^{31}P NMR δ 56.07 in $CDCl_3$). Phospholipases A_2 and C were shown to hydrolyze isomers B

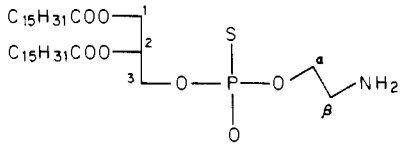
† From the Department of Chemistry, The Ohio State University, Columbus, Ohio 43210. *Received September 13, 1983.* This work was supported by National Institutes of Health Research Grant GM 30327. The NMR facilities used were funded in part by Grants NIH GM 27431 and NSF CHE 7910019. This is paper 7 in the series "Phospholipids Chiral at Phosphorus". For paper 6, see Bruzik & Tsai (1984b). M.-D.T. is an Alfred P. Sloan Fellow, 1983–1985.

[1] Abbreviations: DOC, deoxycholate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DPPsC, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphocholine; DPPsE, 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine; EDTA, ethylenediaminetetraacetate; HMDSA, 1,1,1,3,3,3-hexamethyldisilazane; Mops, 3-(*N*-morpholino)propanesulfonic acid; MPPsE, 1-palmitoyl-*sn*-glycero-3-thiophosphoethanolamine; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; Me_4Si, tetramethylsilane; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/84/0423-1661$01.50/0



DPPsC (1)



DPPsE (2)

and A, respectively, with a high stereospecificity. On the basis of these reactions, pure isomers of chiral DPPsC were isolated and characterized by ^{13}C, ^{1}H, ^{31}P, and ^{14}N NMR. Orr et al. (1982) have also reported stereospecific hydrolysis of 1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoethanolamine (DPPsE) (**2**) by phospholipases A_2 and C. However, pure isomers of DPPsE have not been isolated or characterized. The absolute configuration at phosphorus has not been elucidated for any thiophospholipids.

In this paper we report the preparation and spectral properties of separate diastereomers of DPPsE. The configurations of chiral DPPsC and chiral DPPsE were correlated by transphosphatidylation of DPPsC to DPPsE catalyzed by phospholipase D, which is stereospecific to isomer A of DPPsC. The absolute configurations of chiral DPPsC and chiral DPPsE were then determined by conversion to chiral 1,2-dipalmitoyl-*sn*-glycero-3-[^{18}O]phosphoethanolamine ([^{18}O]-DPPE) followed by ^{31}P NMR analysis.

Materials and Methods

Materials. Phospholipase A_2 (2000–2500 units/mg of protein) from bee venom was isolated from lyophilized whole venom of *Apis mellifera* (Sigma, grade IV) according to the procedure of Shipolini et al. (1971) and assayed as described previously (Bruzik et al., 1983). Phospholipase C from *Bacillus cereus* was purchased from Sigma [type V, suspension in 3.2 M $(NH_4)_2SO_4$ solution, 400–600 units/mg of protein]. Phospholipase D from cabbage was partially purified (1–5 units/mg of protein) according to the procedure of Davidson & Long (1958), as well as obtained from Boehringer Mannheim (0.3 unit/mg of protein). DPPC, DPPE, Triton X-100, sodium deoxycholate, and other biochemicals were purchased from Sigma. $H_2{}^{18}O$ (97.5 atom % ^{18}O) was obtained from Monsanto. Other chemicals were of reagent grade or the highest purity available.

Chromatography. Thin-layer chromatography (TLC) of thiophospholipids was carried out in chloroform/methanol/water (50:50:2, by volume) on precoated plates (0.2 mm, aluminum support, E. Merck silica gel 60 F-254). The R_f values are as follows: DPPsE, 0.75; MPPsE, 0.5. The phospholipids and thiophospholipids were visualized by spraying with phosphomolybdic acid (5 g in 100 mL of ethanol) followed by heating.

Column chromatography of DPPsE was also performed on silica gel columns (Woelm, 40–63-μm particle size) under moderate pressure ($\geq$20 psi). The solvent system used for elution was $CHCl_3/CH_3OH/H_2O$, 50/50/2, unless otherwise specified.

Preparation of Chiral DPPsE. DPPsE(A + B) was synthesized from 1,2-dipalmitoyl-*sn*-glycerol (Bruzik et al., 1983) according to the procedure of Orr et al. (1982), except that toluene was replaced by *p*-xylene. The detailed procedures were also referenced to Eibl (1978) for the synthesis of glycerophospholipids. The yield (74%) was an improvement from the previous report (45%). Hydrolysis of DPPsE by phospholipase C was also carried out as described by Orr et al. (1982). The unreacted DPPsE(B) was isolated in 45% yield (relative to the mixture) after column chromatography. For the hydrolysis by phospholipase A_2, DPPsE(A + B) (184 mg, 0.26 mmol) was dissolved in 2 mL of chloroform (reagent grade) and then mixed with 30 mL of diethyl ether (reagent grade, redistilled). To this solution was added 4 mL of 0.5 M Tris buffer (pH 8.0, containing 0.1 M $CaCl_2$ and 1 mM EDTA) and ca. 200 units of phospholipase A_2. The reaction mixture was stirred at room temperature and the reaction followed by TLC until no further increase in the formation of the product (MPPsE). The reaction mixture was evaporated to dryness and separated by column chromatography. The unreacted DPPsE(A) was isolated in 36% yield (67 mg). The product, MPPsE(B) (^{31}P NMR δ 60.0 ppm in $CDCl_3$, 59.1 ppm in CH_3OD), was acylated to DPPsE(B) as described below. To a solution of MPPsE (100 mg, 0.21 mmol) in dry $CHCl_3$ (15 mL) was added palmitoyl chloride (58 mg, 0.21 mmol) followed by CF_3COOH (17 μL, 26 mg, 0.23 mmol). After being stirred at room temperature for 100 h, the reaction was complete as indicated by TLC. $CaCl_2$ (0.5 mmol) in 1 mL of H_2O was then added to the reaction mixture to precipitate out the unreacted fatty acid. After being stirred for 30 min, the precipitate was filtered and the filtrate extracted with chloroform. The chloroform extract was evaporated to dryness and loaded onto a silica gel column. DPPsE(B) was isolated in ca. 40% yield and characterized by ^{31}P NMR in $CDCl_3/Et_3N$ (94% diastereomeric purity).

Transphosphatidylation of DPPsC. A typical reaction is described as follows. DPPsC (25 mg) was suspended in 95 mL of ethanolamine solution (containing 8% of ethanolamine and 0.04 M $CaCl_2$, adjusted to pH 5.6 with CH_3COOH). After addition of 55 mL of ether and 10 mg of phospholipase D from cabbage, the reaction mixture was stirred at room temperature for a period of time (e.g., 1–2 days). The formation of DPPsE was followed by TLC (chloroform/methanol, 7/3; R_f 0.17 for DPPsC, 0.77 for DPPsE). For more quantitative monitoring of the reaction, the substrates and products were isolated and analyzed by ^{31}P NMR as described below. The ether layer was separated. The aqueous layer was adjusted to pH 4.5 with HCl and extracted with chloroform. The organic fractions were combined, concentrated, loaded onto a silica gel column, and eluted with $EtOH/CHCl_3/H_2O$, 50/50/2. The fractions containing phospholipids were pooled, evaporated to dryness, and analyzed by ^{31}P NMR in CH_3OD. If the reaction was incomplete, the sample was subjected to transphosphatidylation again as described above. In the experiments of Figure 4, the two separate isomers A and B of DPPsC were treated as above in parallel. In other reactions using DPPsC(A + B), the product DPPsE was isolated and characterized as DPPsE(A) by ^{1}H and ^{31}P NMR in $CDCl_3$.

Desulfurization of Thiophospholipids. A typical reaction of DPPsE is described as follows. To a solution of DPPsE (50 mg, 71 μmol) in 1.6 mL of dry dimethoxyethane was added 114 μL of $H_2{}^{18}O$ (113.6 mg, 5.68 mmol, 97.5 atom % ^{18}O] followed with 14.5 μL of Br_2 (284 μmol). The reaction mixture was stirred at room temperature and the reaction monitored by TLC every 5 min after the first 15 min. During the development of TLC plates, the reaction mixture was frozen by dry ice to prevent overreaction. After the reaction was complete (ca. 25 min), excess Na_2SO_3 was added, and the reaction mixture was stirred for a few minutes to quench the reaction. The insoluble material was removed by filtration, and the

filtrate was evaporated to dryness and purified by a silica gel column. The product [^{18}O]DPPE was isolated in 96% yield and characterized by ^{31}P NMR and TLC. It is very important than the reaction was stopped as soon as, or shortly before, it was complete. Otherwise, the isolated [^{18}O]DPPE may have been racemized. The reason for such a racemization (which was not accompanied by an additional isotope exchange) is unclear and has not been further investigated.

Desulfurization of DPPsC mediated by Br_2 was performed as described for DPPsE, and the product [^{18}O]DPPC was isolated in >90% yield. Alternatively, DPPsC (0.13 mmol) in 2.0 mL of dimethoxyethane was added with 200 μL of $H_2{}^{18}O$, 1.3 mmol of 2,6-lutidine, and 0.52 mmol of BrCN. The reaction mixture was stirred at room temperature and monitored as described above. After the reaction was complete (ca. 40 min), the reaction mixture was quenched with excess $NaHSO_3$ and worked up accordingly. The [^{18}O]DPPC was isolated in >80% yield. In all cases, the [^{18}O]DPPC was converted to [^{18}O]DPPE by phospholipase D as described previously (Bruzik & Tsai, 1984b).

Spectral Methods. ^{13}C and ^{1}H NMR were performed on a Bruker WP-200 NMR spectrometer, whereas a Bruker WM-300 was used for ^{31}P NMR. Both spectrometers are equipped with a multinuclear probe. The probe sizes are 5 mm for ^{1}H and 10 mm for ^{31}P and ^{13}C. Deuterium lock was used in all experiments. The chemical shifts for ^{13}C and ^{1}H are referenced to internal Me_4Si, whereas ^{31}P chemical shifts are referenced to external 85% H_3PO_4 at 25 °C. The + signal indicates a downfield shift in all cases.

Results

Preparation of Chiral DPPsE. Orr et al. (1982) have synthesized DPPsE as a diastereomeric mixture and have shown that phospholipases A_2 (from bee venom) and C (from *B. cereus*) hydrolyze a different one of the two isomers specifically. They have, however, not isolated and characterized the separate isomers of DPPsE. Following their procedure, as well as the procedure of Eibl (1978) for the synthesis of glycerophospholipids, we have synthesized DPPsE in a higher yield (74%). ^{31}P NMR analysis in CD_3OD showed two signals as shown in Figure 1a (methanol was chosen as a solvent in this case since most phospholipids are known to be least aggregated in methanol). The unreacted DPPsE isolated from the hydrolysis by phospholipase A_2 (bee venom) was shown to give the upper field signal (60.081 ppm) (Figure 1b,c) and was designated as isomer A. The unreacted DPPsE isolated from the hydrolysis by phospholipase C (*B. cereus*) was shown to give the lower field signal (60.099 ppm) (Figure 1d,e) and was designated as isomer B.

Pure DPPsE(B) was also obtained by acylation of the MPPsE obtained from hydrolysis of phospholipase A_2, as described previously for the preparation of DPPsC(B) (Bruzik et al., 1983). However, since the $-NH_2$ group is also a potential site of acylation under basic conditions, the acylation of MPPsE was carried out by use of acid-catalyst CF_3COOH.

Conversion of DPPsC(A) to DPPsE(A) by Phospholipase D. The configuration of DPPsC and DPPsE (at phosphorus) can indeed be related with each other by the assumption that both phospholipases A_2 and C have the same stereochemical specificity in the hydrolysis of both DPPsC and DPPsE. Thus, phospholipase A_2 hydrolyzes the B isomer of both DPPsC and DPPsE specifically, whereas phospholipase C hydrolyzes the A isomer of both DPPsC and DPPsE specifically.

Another evidence strengthening such a correlation is provided by the transphosphatidylation of DPPsC catalyzed by phospholipase D from cabbage. It has been shown previously

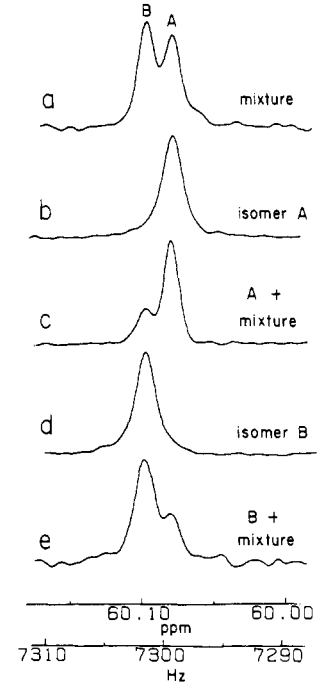


FIGURE 1: ^{31}P NMR spectra (at 121.5 MHz) of DPPsE in CD_3OD: (a) mixture of diastereomers from chemical synthesis; (b) pure isomer A recovered from hydrolysis by phospholipase A_2; (c) isomer A + mixture; (d) pure isomer B recovered from hydrolysis by phospholipase C; (e) isomer B + mixture. NMR parameters were as follows: spectral width 700 Hz, 8K data points, ^{1}H decoupling; 90° pulse, acquisition time 5.8 s, line broadening −0.9 Hz, Gaussian broadening 0.07 Hz, and temperature 40 °C.

(Bruzik & Tsai, 1982, 1984b) that phospholipase D catalyzes transphosphatidylation of chiral [^{18}O]DPPC to chiral [^{18}O]-DPPE with retention of configuration. When DPPsC(A + B) was digested with phospholipase D in the presence of ethanolamine, the unreacted DPPsC was predominantly isomer B as shown by ^{31}P NMR analysis. The product DPPsE isolated from the reaction mixture was shown to be DPPsE(A) by ^{31}P NMR in $CDCl_3$. The results by use of single isomers of DPPsC are shown in Figure 2, which clearly indicates that DPPsC(A) gave DPPsE(A) upon transphosphatidylation (Figure 2a–c) whereas DPPsC(B) did not react under the same conditions (Figure 2d). Thus, phospholipase D from cabbage is specific to the isomer A of DPPsC. If it is assumed that transphosphatidylation proceeds with retention of configuration (as in the case of [^{18}O]DPPC), the fact that DPPsE(A) was obtained reconfirms that DPPsC(A) and DPPsE(A) have the same configuration at phosphorus. On the other hand, if it is assumed that DPPsC(A) and DPPsE(A) have the configuration on the basis of the stereospecificity of phospholipases A_2 and C, the result reconfirms that transphosphatidylation of DPPsC proceeds with retention of configuration. Such correlations are shown in part of Figure 3.

Absolute Configuration of Chiral Thiophospholipids. Recently, we have established the configuration of chiral [^{18}O]DPPE on the basis of ^{31}P NMR analysis (Bruzik & Tsai, 1984a). The configuration of chiral DPPsC and chiral DPPsE can therefore be elucidated by conversion to [^{18}O]DPPE by procedures of known stereochemistry. It has been shown that desulfurization of various nucleoside phosphorothioates in $H_2{}^{18}O$ mediated by bromine, cyanogen bromide, or *N*-bromosuccinimide proceeds with inversion of configuration at phosphorus (Connolly et al., 1982; Lowe et al., 1982, 1983; Potter et al., 1983; Sammons & Frey, 1982; Senter et al., 1983). Such a reaction can therefore be used to correlate the configuration of chiral thiophospholipids with that of [^{18}O]-

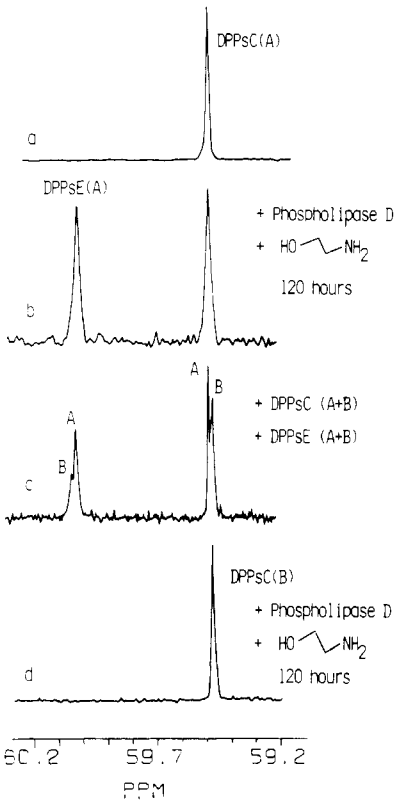


FIGURE 2: Stereospecificity of phospholipase D from cabbage shown by ^{31}P NMR (at 121.5 MHz, CH_3OD): (a) pure DPPsC(A), before reaction; (b) reaction with ethanolamine catalyzed by phospholipase D for 120 h; (c) addition of DPPsC(A + B) and DPPsE(A + B) to (b) to confirm the assignments; (d) DPPsC(B) after the same reaction, with no detectable products. NMR parameters were as follows: spectral width 800 Hz, 16K data points, acquisition time 10.2 s, ^1H decoupling, 90° pulse, line broadening −0.9 Hz, Gaussian broadening 0.07 Hz, and temperature 40 °C.

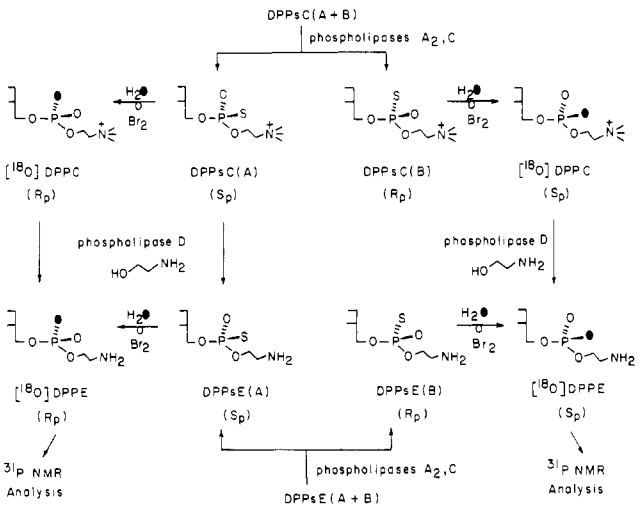


FIGURE 3: Procedures for the configurational analysis of chiral DPPsC and chiral DPPsE.

DPPE. As shown in Figure 3, separate isomers of DPPsE were desulfurized in $H_2{}^{18}O$ in the presence of Br_2 to give two isomers of [^{18}O]DPPE. The configuration of [^{18}O]DPPE was analyzed by ^{31}P NMR following silylation by adding HMDSA (Bruzik & Tsai, 1984a,b). The results, shown in Figure 4, indicated that DPPsE(A) gave (R_P)-[^{18}O]DPPE (Figure 4a) whereas DPPsE(B) gave (S_P)-[^{18}O]DPPE (Figure 4b). The degree of stereospecificity and the percent ^{18}O enrichment are summarized in Table I. On the basis of the assumption that desulfurization proceeds with inversion of configuration, isomer

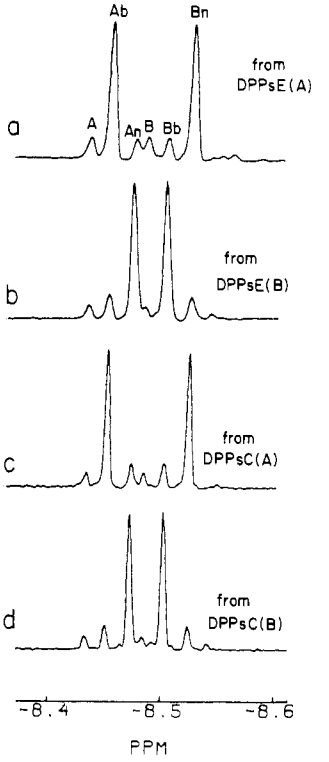


FIGURE 4: ^{31}P NMR spectra (at 121.5 MHz, $CDCl_3$) of the silylated products of chiral [^{18}O]DPPE: (a) (R_P)-[^{18}O]DPPE, from desulfurization of DPPsE(A) mediated by Br_2; (b) (S_P)-[^{18}O]DPPE from DPPsE(B) under the same conditions; (c) (R_P)-[^{18}O]DPPE from Br_2-mediated desulfurization of DPPsC(A) followed by transphosphatidylation; (d) (S_P)-[^{18}O]DPPE from DPPsC(B) under the same conditions. NMR parameters were as follows: spectral width 500 Hz, ^1H decoupling, 90° pulse, acquisition time 8 (a and b) and 16 s (c and d), temperature 25 °C, line broadening −0.9 Hz, and Gaussian broadening 0.07 Hz. The species A, Ab, An, B, Bb, and Bn are as defined previously (Bruzik & Tsai, 1982, 1984a,b).

Table I: Quantitative Results of Desulfurization[a]

compd	isomeric composition % S_P	% R_P	reagent	[^{18}O]DPPE % ^{18}O	% R_P	% S_P
DPPsE (mixture)			Br_2	70	52	48
DPPsE	97	3	Br_2	87	87	13
DPPsE	3	97	Br_2	92	15	85
DPPsC (mixture)			Br_2	85	50	50
DPPsC	85	15	Br_2	92	84	16
DPPsC	5	95	Br_2	93	15	85
DPPsC	85	15	CNBr	68	71	29
DPPsC	5	95	CNBr	91	20	80

[a] Isotopic and diastereomeric enrichments were determined by ^{31}P NMR. The estimated error in the measurement of peak integrals is <5%.

A of DPPsE should be S_P and isomer B of DPPsE should be R_P, as shown in Figure 3.

Although the configuration of chiral DPPsC can now be assigned on the basis of the correlation between DPPsE and DPPsC, we have carried out an independent determination. As shown in Figure 3, separate isomers of DPPsC were desulfurized, in the presence of Br_2 or CNBr, to give separate diastereomers of chiral [^{18}O]DPPC. The configuration of [^{18}O]DPPC was determined by conversion to [^{18}O]DPPE catalyzed by phospholipase D (with retention of configuration) followed by ^{31}P NMR analysis. As shown in Figure 4c,d, DPPsC(A) gave (R_P)-[^{18}O]DPPE whereas DPPsC(B) gave (S_P)-[^{18}O]DPPE. The quantitative results are also summa-

Table II: Summary of ^{31}P Chemical Shifts

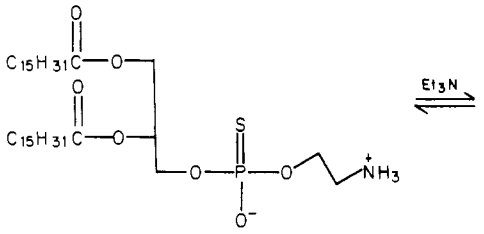
condition[a]	(S_P)-DPPsE	(R_P)-DPPsE[b]	DPPE
$CDCl_3$	59.95	59.82 (−0.13)	0.74
$CDCl_3$ + Et_3N	59.29	59.13 (−0.16)	0.78
$CDCl_3$ + CF_3COOH	62.5[c]	62.5[c]	−1.11
CH_3OD	60.081	60.099 (0.018)	1.46

[a] The base (Et_3N) and acid (CF_3COOH) added were in excess, ca. 3–5 equiv. Further addition caused very small changes. [b] Numbers in parentheses are differences between the two diastereomers. [c] In the presence of acid, peaks are broad, and the two isomers cannot be resolved.

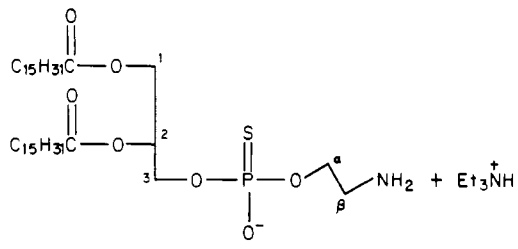
rized in Table I. The results indicate that DPPsC(A) should be S_P whereas DPPsC(B) should be R_P, as shown in Figure 3.

Spectral Properties of Chiral DPPsE. Before chiral DPPsE can be applied to investigate various biochemical problems, it is necessary to understand their properties in terms of the similarity between DPPsE and DPPE, and the difference between the two diastereomers of DPPsE. These properties were investigated by ^{31}P, ^{13}C, and ^1H NMR.

(1) ^{31}P NMR. Although the methanol solution of DPPsE gave sharp ^{31}P NMR signals as shown in Figure 1 (half width ≤2 Hz), the compound had a low solubility, and its spectrum contained two ill-resolved peaks (0.018 ppm). In chloroform, the solubility and separation are larger (Orr et al., 1982), but signals are also broader due to aggregation. However, we found that addition of one or two drops of triethylamine to the DPPsE solution in $CDCl_3$ caused a dramatic increase in solubility, in resolution (half width <2 Hz), and in the separation of the two peaks (0.16 ppm), as shown in Figure 5. The diasteromeric purity of the two separate isomers (Figure 5b,d) can be quantitated as 97% in both cases. Such changes are apparently due to the change from the zwitter ionic form of DPPsE (**3**) to the anionic form (**4**) (localization of the double bond at P=S is an assumption without proof):



3



4

Table II summarizes the ^{31}P chemical shifts of (R_P)-DPPsE, (S_P)-DPPsE, and DPPE under various conditions. The fact that DPPsE resonates ca. 55–60 ppm downfield from DPPE is consistent with the ^{31}P NMR property of other phosphorothioates (Tsai, 1984) and provides a useful property for biochemical applications. The observation that DPPsE is shifted downfield whereas DPPE is shifted upfield upon protonation of the phosphate group is also consistent with the property of nucleoside phosphorothioates (Jaffe & Cohn, 1978).

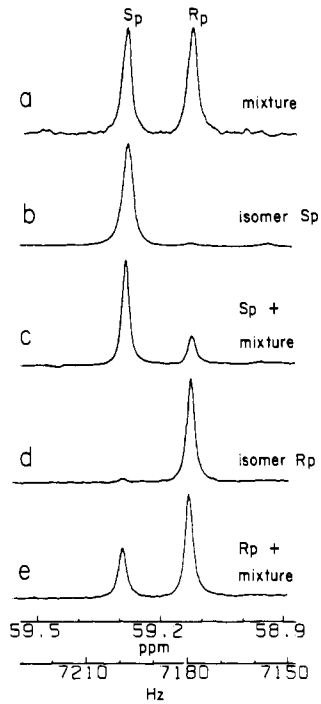


FIGURE 5: ^{31}P NMR spectra (at 121.5 MHz) of DPPsE in $CDCl_3$ containing 3-5 equiv of triethylamine: (a) mixture of diastereomers from chemical synthesis; (b) S_P isomer; (c) S_P isomer + mixture; (d) R_P isomer; (e) R_P isomer + mixture. NMR parameters were as follows: spectral width 1400 Hz, 8K data points, acquisition time 2.9 s, ^1H decoupling, 90° pulse, line broadening 1.0 Hz, and temperature 25 °C.

(2) ^{13}C NMR. The ^{13}C NMR spectrum of DPPE in $CDCl_3/CD_3OD/D_2O$ (50:50:15, by volume) has been reported recently (Murari et al., 1982). In Table III, we compare the ^{13}C chemical shifts and $J_{^{13}C-^{31}P}$ of (R_P)-DPPsE, (S_P)-DPPsE, and DPPE under three different conditions: $CDCl_3/CD_3OD$ (1:1, by volume), $CDCl_3$ + Et_3N, and $CDCl_3$ + [$^2H_5$]pyridine. The results indicate that the $J_{^{13}C-^{31}P}$ values are the same within experimental errors for DPPsE and DPPE. Since $J_{^{13}C-^{31}P}$ is known to be sensitive to the conformation of molecules (Lapper & Smith, 1973; Smith et al., 1975; Alderfer & Ts'o, 1977), DPPE and DPPsE should assume similar conformations in solution. In addition, DPPsE and DPPE showed similar solvent dependence in chemical shifts. On the other hand, the two diastereomers of DPPsE showed a small difference in the chemical shifts of C_3 (in $CDCl_3/CD_3OD$) (Figure 6a), C_2 (in $CDCl_3$ + Et_3N) (Figure 6c), and even in one of the carbonyl carbon (most likely at the 2-carboxyl ester) (in $CDCl_3$ + pyridine) (Figure 6b).

(3) ^1H NMR. Akutsu & Kyogoku (1977) have used ^1H NMR to analyze the conformation of phosphatidylethanolamine and related compounds. At 200 MHz, in a solution of $CDCl_3$ containing Et_3N, we were able to resolve all protons in the head group region of DPPE: (ethanolamine chain) δ 2.89 (t, J_{vic} = 5.9 Hz, β-CH_2N), 3.90 (q, $^3J_{HP}$ = J_{vic} = 5.7 Hz, α-CH_2); (glycerol backbone) δ 4.18 (dd, J_{gem} = 11.9 Hz, J_{vic} = 6.6 Hz, 1-CH_2O), 4.41 (dd, J_{gem} = 12.0 Hz, J_{vic} = 3.4 Hz, 1-CH_2O), 5.25 (m, 2-CHO), 4.0 (t, $^3J_{HP}$ = J_{vic} = 5.7 Hz, 3-CH_2OP). The complete assignments for DPPsE(A + B) under the same conditions are as follows: (ethanolamine chain) δ 3.12 (m, β-CH_2N), 4.1 (m, α-CH_2); (glycerol backbone) δ 4.17 (dd, J_{gem} = 12.0 Hz, J_{vic} = 6.4 Hz, 1-CH_2O), 4.38 (dd, J_{gem} = 12.0 Hz, J_{vic} = 2.9 Hz, 1-CH_2O), 5.25 (m, 2-CHO), 4.1 (m, 3-CH_2OP); (alkyl chain) δ 2.3 (m, 2-CH_2), 1.6 (m, 3-CH_2), 1.26 (br, methylene C_4–C_{15}), 0.88 (t, J_{vic} = 6.7 Hz, terminal CH_3). For the coupling constants that can be re-

Table III: ^{13}C Chemical Shifts (δ) of DPPsE and DPPE[a]

	$CDCl_3/CD_3OD$		$CDCl_3/[^2H_5]$pyridine		$CDCl_3/Et_3N$	
	DPPE	DPPsE	DPPE	DPPsE	DPPE	DPPsE
ethanolamine chain						
β-CH_2N	41.34	41.09	40.3	40.58	40.85	41.38
$^3J_{CP}$	4.8	6.0				4.9
α-CH_2OP	62.23	62.42	62.03	62.47	*c*	*d*
$^2J_{CP}$	5.0	5.4	5.3			
glycerol backbone						
1-CH_2O	63.29	63.34	62.41	62.47	62.50	62.64
2-CHO	71.24	71.18	70.18	70.27	70.29	70.257 (S_P), 70.323 (R_P)
$^3J_{CP}$	7.9	8.8	7.4		7.5	8.3
3-CH_2OP	64.51	64.85 (R_P), 64.95 (S_P)	63.82	64.98	*c*	*d*
$^2J_{CP}$	5.0	4.7				
carbonyl						
C=O	174.56	174.59	172.84	173.522	172.83	173.38
	174.93	174.96	173.11	173.753 (S_P), 173.797 (R_P)	173.13	173.75
acyl chains[b]						
2-CH_2	34.83	34.84	34.09	34.26	34.14	34.24
	34.67	34.69	33.90	34.05	33.95	34.05
3-CH_2	32.48	32.46	31.72	31.81	31.75	31.82
C_4-C_{13}	29-30	29-30	29-30	29-30	29-30	29-30
14-CH_2	25.47	25.44	24.74	24.78	24.74	24.79
15-CH_2	23.16	23.13	22.45	22.53	22.48	22.54
16-CH_3	14.27	14.24	13.83	13.91	13.85	13.92

[a] Spectral parameters are as described in the legend of Figure 6. [b] The numbering of acyl chains starts from the carboxyl carbon as number 1. [c] δ 63.22 or 63.74. [d] δ 64.45 or 65.09.

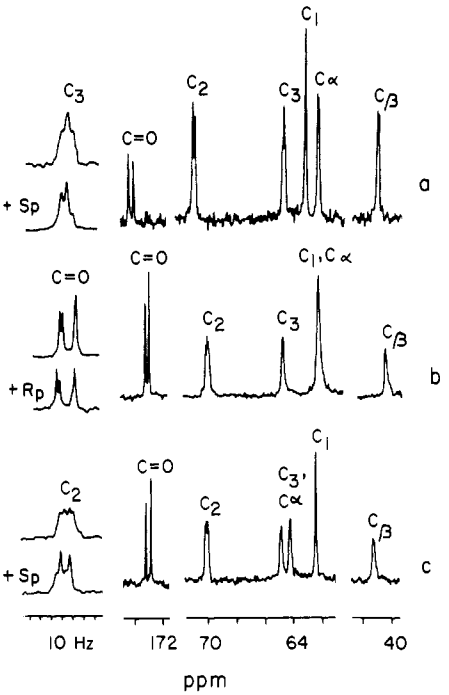


FIGURE 6: ^{13}C NMR spectra (at 50.3 MHz) of DPPsE($R_P + S_P$) for carbons in the glycerol backbone, the ethanolamine chain, and the carbonyl groups: (a) 120 mg in 0.9 mL of $CDCl_3$ + 0.9 mL of CD_3OD; (b) 120 mg in $CDCl_3$ + 2 equiv of $[^2H_5]$pyridine; (c) 120 mg in $CDCl_3$ + 2 equiv of triethylamine. The expanded signals on the left-hand side are the resonances that can be resolved for the two diastereomers. A single diastereomer was then added to the mixture such that the signals can be assigned. NMR parameters were as follows: spectral width 11 kHz, acquisition time 0.74 s, acquisition delay 0.8 s, 45° pulse, broad-band ^1H decoupling, temperature 37 °C, and resolution 1.36 Hz/point.

solved, there is little difference between DPPsE and DPPE within experimental errors. This again supports that DPPsE and DPPE assume similar conformation in solution. The two diastereomers were not resolvable by ^1H NMR.

Discussion

The results presented in this paper are significant in several respects. (a) The desulfurization reaction provides a simpler way to synthesize chiral [^{18}O]DPPE and chiral [^{18}O]DPPC, which were synthesized previously with approximately the same isotopic purity and diastereomeric purity by more elaborate procedures (Bruzik & Tsai, 1982, 1984a,b). In addition, if an oxygen isotope is introduced in the synthesis of DPPsC or DPPsE, then chirally labeled [^{17}O,^{18}O]DPPC and [^{17}O,^{18}O]DPPE can be obtained. These compounds are useful in biochemical studies.

(b) Elucidation of the absolute configuration of chiral DPPsE and chiral DPPsC, on the basis of a safe assumption that desulfurization proceeds with inversion of configuration (Connolly et al., 1982; Lowe et al., 1982, 1983; Potter et al., 1983; Sammons & Frey, 1982; Senter et al., 1983), makes it possible to interpret the results of biochemical study by use of chiral thiophospholipids in a stereochemical way. For example, it is now clear that phospholipase A_2 is specific to the R_P isomer of DPPsC and DPPsE whereas phospholipases C and D are specific to the S_P isomer. In addition, other types of chiral thiophospholipids and chiral lysothiophospholipids that may be synthesized in the future can be correlated with DPPsE or DPPsC for configurational determination.

(c) The finding that phospholipase D is specific to the S_P isomer of DPPsC in transphosphatidylation and that transphosphatidylation of DPPsC proceeds with retention of configuration, together with the finding described in the preceding paper (Bruzik & Tsai, 1984b), provides a complete feature on the stereochemical mechanism of phospholipase D. Such results are particularly valuable since the enzyme has not been purified to homogeneity, and kinetic study is difficult due to the lack of pure enzyme and the heterogeneity of the reaction mixture.

(d) Preparation and characterization of separate diastereomers of DPPsE, in combination with the previous work on chiral DPPsC (Bruzik et al., 1983), open up a number of

possibilities for biochemical and biophysical applications, particularly in studying the structure and function of the head group of phospholipids in membranes. Thiophospholipids are a good model of phospholipids since they could assume a conformation similar to that of natural phospholipids in solution, as suggested by this and the previous (Bruzik et al., 1983) papers. In addition, thiophospholipids can form lipid bilayers resembling natural phospholipids (Vasilenko et al., 1982; Tsai et al., 1983). On the other hand, the chirality at phosphorus provides a new probe for conformational study, and the two diasteromers are distinguishable both in solution and in lipid bilayers (Tsai et al., 1983). In two recent studies, a diastereomeric mixture of thiophospholipids was employed (Vasilenko et al., 1982; Hui et al., 1983). Since separate diastereomers are easily obtainable, we strongly suggest that separate diastereomers be used in future studies.

Acknowledgments

We are indebted to Dr. K. Bruzik of this laboratory for some initial study on the stereospecificity of phospholipase D and to Judy Hart of this laboratory for purification of phospholipases A_2 and D.

Registry No. **1** (isomer A), 82482-78-8; **1** (isomer B), 82482-77-7; **2** (isomer A), 81904-63-4; **2** (isomer B), 81904-62-3; (*R*)-[^{18}O]DPPC, 80548-30-7; (*S*)-[^{18}O]DPPC, 88945-76-0; (*R*)-[^{18}O]DPPE, 80548-28-3; (*S*)-[^{18}O]DPPE, 88035-45-4; (*R*)-MPPsE, 88945-77-1; phospholipase D, 9001-87-0; bromine, 7726-95-6; cyanogen bromide, 506-68-3; palmitoyl chloride, 112-67-4.

References

Akutsu, H., & Kyogoku, Y. (1977) *Chem. Phys. Lipids 18*, 285–303.

Bruzik, K., & Tsai, M.-D. (1982) *J. Am. Chem. Soc. 104*, 863–865.

Bruzik, K., & Tsai, M.-D. (1984a) *J. Am. Chem. Soc. 106*, 747–754.

Bruzik, K., & Tsai, M.-D. (1984b) *Biochemistry* (preceding paper in this issue).

Bruzik, K., Gupte, S. M., & Tsai, M.-D. (1982) *J. Am. Chem. Soc. 104*, 4682–4684.

Bruzik, K., Jiang, R.-T., & Tsai, M.-D. (1983) *Biochemistry 22*, 2478–2486.

Chupin, V. V., Vasilenko, I. A., Predvoditeleu, D. A., Sercbrennikova, G. A., & Evstigneeva, R. P. (1979) *Dokl. Akad. Nauk. SSSR 248*, 235–237.

Connolly, B. A., Eckstein, F., & Füldner, H. H. (1982) *J. Biol. Chem. 257*, 3382–3384.

Davidson, F. M., & Long, C. (1958) *Biochem. J. 69*, 458–466.

Eckstein, F. (1983) *Angew. Chem., Int. Ed. Engl. 22*, 423–439.

Eibl, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4074–4077.

Hui, S. W., Boni, L. T., Stewart, T. P., & Isac, T. (1983) *Biochemistry 22*, 3511–3516.

Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652–657.

Lapper, R. D., & Smith, I. C. P. (1973) *J. Am. Chem. Soc. 95*, 2880–2884.

Lowe, G., Tansley, G., & Cullis, P. M. (1982) *J. Chem. Soc., Chem. Commun.*, 595–598.

Lowe, G., Sproat, B. S., Tansley, G., & Cullis, P. M. (1983) *Biochemistry 22*, 1229–1236.

Murari, R., El-Rahman, M. M. A. A., Wedmid, Y., Parthasarathy, S., & Baumann, W. J. (1982) *J. Org. Chem. 47*, 2158–2163.

Nifant'ev, E. E., Predvoditelev, D. A., & Alarkon, Kh. Kh. (1978) *Z. Org. Khim. 14*, 56–63.

Orr, G. A., Brewer, C. F., & Heney, G. (1982) *Biochemistry 21*, 3202–3206.

Potter, B. V. L., Connolly, B. A., & Eckstein, F. (1983) *Biochemistry 22*, 1369–1377.

Sammons, D., & Frey, P. A. (1982) *J. Biol. Chem. 257*, 1138–1141.

Senter, P. D., Eckstein, F., Mülsch, A., & Böhme, E. (1983) *J. Biol. Chem. 258*, 6741–6745.

Shipolini, R. A., Callewaert, G. L., Cottrell, R. C., Doonan, S., Vernon, C. A., & Banks, B. E. C. (1971) *Eur. J. Biochem. 20*, 459–468.

Smith, I. C. P., Jennings, H. J., & Deslauriers, R. (1975) *Acc. Chem. Res. 8*, 306–313.

Tsai, M.-D. (1984) in ^{31}P *NMR: Principles and Applications* (Gorenstein, D., Ed.) pp 175–197, Academic Press, New York.

Tsai, M.-D., Jiang, R.-T., & Bruzik, K. (1983) *J. Am. Chem. Soc. 105*, 2478–2480.

Vasilenko, I., DeKruijff, B., & Verkleij, A. J. (1982) *Biochim. Biophys. Acta 685*, 144–152.